

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

261

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>C12Q 1/68</b>	<b>A1</b>	(11) International Publication Number: <b>WO 98/37242</b>
		(43) International Publication Date: 27 August 1998 (27.08.98)

(21) International Application Number: PCT/US98/03726

(22) International Filing Date: 23 February 1998 (23.02.98)

(30) Priority Data:  
60/038,796 24 February 1997 (24.02.97) US(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application  
US 60/038,796 (CIP)  
Filed on 24 February 1997 (24.02.97)

(71) Applicant (for all designated States except US): TM TECHNOLOGIES, INC. [US/US]; 82 Cummings Park, Woburn, MA 01801 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): LANE, Michael, J. [US/US]; 3044 Claybourne Lane, Baldwinsville, NY 13027 (US). BENIGHT, Albert, S. [US/US]; 1630 Valley View Drive, Schaumburg, IL 60193 (US). FALDASZ, Brian, D. [US/US]; 4 Bellevue Terrace, Maynard, MA 01754 (US).

(74) Agents: DECONTI, Giulio, A., Jr. et al.; Lahive &amp; Cockfield, LLP, 28 State Street, Boston, MA 02109 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published***With international search report.**Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: PROCESS FOR SELECTING ANTI-SENSE OLIGONUCLEOTIDES

## (57) Abstract

Methods for identifying a site on a nucleic acid sequence having a desired free energy variability are disclosed. The methods are useful for, e. g., selecting anti-sense oligonucleotides, e.g., for therapeutic use.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## PROCESS FOR SELECTING ANTI-SENSE OLIGONUCLEOTIDES

### Field of the Invention

This invention relates to a means of selecting nucleotide sequences having a  
5 selected value for free energy variance. The methods allow for selecting anti-sense  
oligonucleotides, for use e.g., as pharmacological agents, from a set of candidates, e.g.,  
those provided by a given nucleic acid, e.g., an mRNA.

### Background of the Invention

10 Antisense therapy involves the administration of exogenous oligonucleotides  
that bind to a target nucleic acid, typically an RNA molecule, located within cells. The  
term antisense is so given because the oligonucleotides are typically complementary to  
mRNA molecules ("sense strands") which encode a cellular product. The ability to use  
anti-sense oligonucleotides to inhibit expression of mRNAs, and thereby to inhibit  
15 protein expression *in vivo*, is well documented. However, selection of an appropriate  
complimentary oligonucleotide (or oligonucleotides) to a given mRNA is not always  
simple (see, e.g., Crooke, S.T. *FASEB J.* 7: 533-539 (1993), incorporated herein by  
reference). Anti-sense agents typically need to continuously bind all target RNA  
molecules so as to inactivate them or alternatively provide a substrate for endogenous  
20 ribonuclease H (Rnase H) activity. Sensitivity of RNA/oligonucleotide complexes,  
generated by the methods of the present invention, to Rnase H digestion can be  
evaluated by standard methods (see, e.g., Donia, B. P., et al., *J. Biol. Chem.* 268  
(19):14514-14522 (1993); Kawasaki, A. M., et al., *J. Med. Chem.* 6(7):831-841 (1993),  
incorporated herein by reference).

### Summary of the Invention

Prior art methods do not provide efficient means of determining which  
complimentary oligonucleotides to a given mRNA will be useful in an application.  
Shorter (15-200) base anti-sense molecules are preferred in clinical applications. In fact,  
30 a minimum of 15 base anti-sense oligonucleotides is preferred. The invention includes  
methods for selecting desired anti-sense oligonucleotides from the set of candidates  
provided by any given nucleic acid, e.g., an mRNA. In particular, the invention  
provides a means of determining desired, e.g., sequence positions, e.g., those which  
present a desired level of free energy variations on the mRNA to design anti-sense  
35 oligonucleotides against thus reducing the empiricism currently employed.

- 2 -

In one aspect, the invention features a method of identifying a site on a nucleic acid sequence having high free energy variability. This allows determination of sites which are preferred for oligonucleotide, e.g., antisense, binding. The method includes some or all of the following steps:

- 5       providing a nucleotide sequence, e.g., sequence from a target gene;  
      casting the nucleotide sequence as the free energy as a function of base pair position;  
      calculating the free energy of X windows centered on a base pair for a plurality of base pairs from the nucleotide sequence for every, or at least a plurality of window  
10    sizes between 2 and Y, where Y is an integer between 3 and 1,000, more preferably between 2 and 100;  
      for each window size, constructing a free energy distribution along the sequence, preferably normalizing the distribution to a standard scale (to account for the fact that the free energy is proportional to window size) (this calculation gives the results which  
15    can be plotted as shown in Figure 1);  
      finding the mean normalized free energy values for all windows for each base pair position (this gives results which can be plotted as in Figure 2. It also represents the "carrier");  
      subtracting the mean value for a position and provide the deviation from the  
20    mean of each base position to determine those sequence which show high variability. The results can be plotted as in Figure 3 (point "a" in Figs 2 and 3 corresponds to high variability).
- In certain embodiments, free energy values are calculated for a plurality of window sizes at at least Z percent of the base of the nucleotide sequences, wherein Z is  
25    at least 5, 10, 20, 30, 40, 50, 60, 70, 80, or 90% of the base pairs of the nucleotide sequence.

- In another embodiment, the invention provides a method of identifying an optimized ligand binding site on a nucleic acid sequence. The method includes the steps of providing a nucleic acid sequence; calculating a free energy value for at least two  
30    window sizes at each of a plurality of base pairs of the nucleic acid sequence; normalizing the free energy values for each window size at each base pair to a standard scale; and calculating a deviation of each normalized free energy value at a base pair from a mean normalized free energy value at the base pair; and selecting a base pair at which a large deviation from the normalized free energy value is calculated, relative to  
35    at least one other base pair; such that an optimized ligand binding site on the nucleic acid sequence is identified.

- 3 -

In certain embodiments, free energy values are calculated for a plurality of window sizes at at least Z percent of the base of the nucleotide sequences, wherein Z is at least 5, 10, 20, 30, 40, 50, 60, 70, 80, or 90% of the base pairs of the nucleotide sequence.

5 In certain embodiments, free energy values are calculated for at least N window sizes at each of the plurality of base pairs, wherein N is at least 2, 5, 10, 15, 20, 30, 40, or 50 window sizes.

In another aspect, the invention provides for a method for determining preferred anti-sense sequence compliments within a predefined RNA sequence; these are generally  
10 high variability sequences. (As used herein high variability can be a relative parameter, e.g., relative to other variability in the sequence. Alternatively it can be relative to a predefined value).

In another aspect, the invention provides for sets (e.g., sets of 2, 3, 4 or more) of sequences, e.g., anti-sense oligonucleotides, of an optimal duplex free energy or  
15 variability but variable length at the sites of anti-sense candidates within candidate regions.

In another aspect, the invention provides sets of isoenergetic, or isovaryable, oligonucleotides, e.g., anti-sense candidates of a set length within a candidate region.

In yet another aspect, the invention provides for establishing oligonucleotides,  
20 (e.g., sets of 2, 3, 4 or more) oligonucleotides, e.g., anti-sense oligonucleotides, of a preselected melting temperature,  $T_m$  within candidate regions.

Generally, the method allows for identification, choosing, and matching of sequences with desired free energy variability characteristics.

Methods of the invention can be used for any of the following:

25 Determining the best anti-sense candidate regions, or sub-sequences, within any given anti-sense target. Such sequences exhibit wide variation in average energy as a function of increasing length.

Designing desirable attributes such as  $T_m$ , free energy and length coupled with sequence composition to arrive at the best anti-sense oligonucleotide candidates 10-200  
30 bases in length including the pre-identified candidate regions.

Providing compositions of sequence which display the identified variation in sequence composition with changing (e.g., increasing) window size.

Any method of the invention can include providing a sequence, e.g., by synthesizing (by chemical or biochemical methods), or by placing in a reaction mixture  
35 which includes a carrier, e.g., a liquid, e.g., water.

- 4 -

Brief Description of the Drawings

Figure 1 is a plot of normalized energy as a function of window size and position along a representative DNA sequence.

5 Figure 2 is an overlaid plot of the data shown in Figure 1.

Figure 3 is a plot of the variability of energy distributions along the representative DNA sequence.

10 Detailed Description of the Invention

The invention provides a method(s) for determining base position(s) on a preselected mRNA sequence where best hybridization of an oligonucleotide will occur. Note that the mRNA may be a pre-mRNA (hnRNA) thus containing untranscribed regions to be spliced out and that included in this mRNA/pre-mRNA are a variety of control sequences which allow binding of various cellular components.

15

For example, if one were to approach the problem of anti-sense design randomly on, for example, a 1000 base target mRNA molecule, then one could pick a set length oligonucleotide, e.g., 30 bases, and synthesize a thirty-mer starting at position 1 of the mRNA and complimentary to positions 1-30 of the mRNA, followed by synthesis of a second thirty-mer starting at position 2 and ending at position 31. This iterative process of synthesis followed to its conclusion results in  $[1000 \text{ base mRNA} - 2(30 \text{ base anti-sense length}) + 2] = 942$  thirty base anti-sense oligonucleotides. Similarly, of course one might also select nineteen-mers as the optimal length resulting in  $[1000 \text{ base mRNA} - 2(19 \text{ base anti-sense length}) + 2] = 964$  nineteen-base anti-sense oligonucleotides.

20

25

In fact, one could synthesize all such complimentary oligonucleotides of length less than the mRNA length and try to inhibit protein synthesis with each in an attempt to find the best anti-sense oligonucleotide for a given mRNA. However, in practice this approach would be an enormous undertaking. Clearly the process of selecting an anti-sense oligonucleotide of length suitable for large scale use as a pharmaceutical while showing *in vivo* activity would be simplified by identifying the "best" mRNA sequence position to target an anti-sense oligonucleotide against.

30

- 5 -

The method is described below with reference to data from a representative target nucleic acid sequence (LDH M72545, base positions from 64-924; the sequence is available through GENBANK).

5 The algorithm for determining relatively "reactive" sites along genomic DNA is based on a representation of duplex DNA in terms of its sequence dependent melting free-energy. This provides DNA sequence as energy contours, that when scrutinized in the proper way, can lead to direct determination of specific sites that are optimum for targeting by anti-sense therapeutic agents.

10 There are six steps to the current method with at least one step (4) being considered optional:

15 (1) Free-Energy Representation of DNA Sequences: For a DNA sequence comprised of N base pairs (bps), each bp i can be assigned a melting free-energy value,  $\Delta G_i$ ,

$$\Delta G_i = \Delta G^{H-B}_i + (\Delta G^{S}_{i,i-1} + \Delta G^{S}_{i,i+1})/2$$

20 Where  $\Delta G^{H-B}_i$  is the free-energy of hydrogen bonding that typically can take on only two values (for A-T or G-C type bps) and  $\Delta G^{S}_{i,i-1} + \Delta G^{S}_{i,i+1}$  are the nearest-neighbor sequence dependent stacking free-energies for the stacking interactions between bp i and bps i+1 and i-1. Utilizing this equation each bp can be assigned a free-energy of melting.

25 (2) Construction of Free-Energy Windows: In this procedure, windows of bps containing from 2 to 200 bps are individually examined. For each window size, starting at bp 1, the added free-energy of the bps in the window are summed and plotted as the first point. The window is then moved over one bp and the free-energy of the new window that contains the free-energy of a new bp and not the free-energy of the first bp  
30 of the previous window and all the intervening bps, is determined. The process is continued until the last window reaches the end of the DNA sequence under consideration. Formally for each window size,  $j = 10-42$  bps starting at bp  $s = 1$ ,  $N-j+1$  the free-energy of each window is given by,

35 
$$\Delta G_j^w = \sum_{i=s, j+s-1} (\Delta G_i)$$

- 6 -

Thus, plotting the values of  $\Delta G_j^w$  vs bp position  $s$  results in an energy contour for that particular window size,  $j$ . Since the magnitude of  $\Delta G_j^w$  increase with the size of  $j$ , relative features of energy contours constructed for different window sizes are difficult to compare directly.

5

(3) Direct Comparisons of Energy Contours Constructed with Different Window Sizes:

To facilitate such a direct comparison the values of  $\Delta G_j^w$  determined for different values of  $j$  are normalized relative to the maximum free-energy difference of any two windows of size  $j$ . Thus the normalized free-energy for each window is given by

10

$$\langle \Delta G_j^w \rangle = | (\Delta G_j^w - \Delta G_j^w (\min)) / (\Delta G_j^w (\max) - \Delta G_j^w (\min)) |$$

Where  $\Delta G_j^w (\max)$  and  $\Delta G_j^w (\min)$  are the maximum minimum and free-energies observed for all the windows along the sequence of size  $j$ . Now the free-energy contours

15

constructed with different window sizes consist of a distribution of relative free-energies with values between 0 and 1 vs bp position.

Figure 1 is a plot of normalized energy as a function of window size and position along the representative DNA sequence (LDH M72545, base positions from 64-924).

The window size was varied over a range from 10 to 42 for each position, and the energy profile for each base position and window size was plotted.

20

(4) [Optional step] Overlapping Energy Contours Constructed with Different Window Sizes:

A more direct comparison of these energy contours is to "overplot" (e.g., plot one data set over another) them as shown in Fig. 2. Features of the distribution of melting stability are clearly apparent and apparently only slightly dependent on window size over the range examined. Regions of lowest magnitude are the least stable while regions of highest magnitude are the most stable. Although the same general features are observed on all the distribution function shown in Figure 2, there are small deviations (on the order of 10-20%) about what appears to be the "average" shape of the distribution. These distribution directly reveal the contributions of hydrogen bonding and nearest-neighbor stacking to DNA stability. The prominent features of the distribution are generally determined by the amount of A-T or G-C type bps in the sequence. For example, the peaks in the overlaid plots of Fig. 2 depict regions relatively higher in G-C percentage. The converse is true for the "valleys," which reveal a larger percentage of A-T type base pairs in that region. Because of the greater relative energy

25

30

35



- 7 -

of the peaks as compared to the valleys, the effect of window size is more pronounced at the peaks.

(5) Deviations from the Window Size Average Reveal Targetable Regions: The superimposed "noise" or deviations from the mean behavior of the distribution for the different window sizes seen in Fig. 2 reveals the influence of nearest-neighbor stacking on DNA stability. It is this noise pattern that can be isolated. To better examine this component of the distribution functions, the average over all normalized energies determined for each window size are determined at each bp position,  $s$ . That is.

$$\langle \Delta G^w \rangle_{ave}(s) = \sum_j \Delta G_j^w(s) N_w$$

where  $N_w$  is the number of window sizes. Now the differences.

$$\delta \langle \Delta G^w \rangle_{ave}(s) = \langle \Delta G^w \rangle_{ave}(s) - \langle \Delta G_j^w \rangle(s)$$

are determined and plotted vs sequence position for each window size as shown in Fig. 3. The result is a "noise" pattern with most values between -0.20 and +0.20 centering around 0 along the bp position. Notably, several regions emerge from this pattern which display larger range than the preselected noise criteria. These regions are clearly seen in Fig. 3 (e.g., the point labelled "A" in Figure 3 has large variability) and display the highest variability in sequence dependent stability with changes in window size and after scaling the values for the entire sequence set as described above. These are the desired targets for sequence specific anti-sense therapeutics.

(6) Selection of Sequences: The 200 base sequences (other lengths, e.g., 150, 100, or 50 bases, can also be used), 100 to either side of the "variational maxima" seen on the plots of  $\delta \langle \Delta G^w \rangle_{ave}(s)$  vs  $s$  ( $\delta \langle \Delta G^w \rangle_{ave}(s)$ ) are identified from the mRNA sequence and subjected to further examination. While these 200-mers could be used as anti-sense oligonucleotides immediately it is more desirable to use smaller oligomers comprising, e.g., approximately 50, 40, 30 or fewer bases that are subsequences of the selected 200-mer. Optimal anti-sense candidate oligomers within the 200-mer will contain a 2-10 bp more stable region flanked by relatively unstable regions.

In some applications it may be desirable to select sets of anti-sense oligonucleotides all with a pre-defined optimal duplex free-energy but with different

- 8 -

variable lengths. This is done by scanning the energetic distribution of the 200 bp region and determining the various pieces from 15 to 30 bps in length that have the same calculated free-energy of stability.

5 In other applications it may be desirable to select sets of isoenergetic anti-sense candidates of a given length. This is done by scanning the energetic distribution of the 200 bp region and determining the various pieces of a given length that have the same calculated free-energy.

10 In other applications it may be desirable to choose anti-sense oligonucleotides of a preselected melting temperature,  $T_m$ . This can be done using the formula,

$$T_m = (\Delta H_D + \Delta H_{nuc}) / \Delta S_D + \Delta S_{nuc} + \ln(\alpha C_T)$$

15 Where  $\Delta H_D$  and  $\Delta S_D$  are the calculated melting enthalpy and entropy for the particular sequence.

The entropy of nucleation is  $\Delta S_{nuc}$  and is regarded as a constant for a particular type of target in our equational formulation. That is, it does not depend on oligomer  
20 length. In contrast, the enthalpy of duplex nucleation,  $\Delta H_{nuc}$  is primarily electrostatic in nature and therefore depends on sequence length, G-C percentage and salt concentration. The total strand concentration is  $C_T$  and  $\alpha$  is a factor that properly accounts for sequence degeneracies in association of the oligomers. Overall, stability of the chose oligomers can therefore be adjusted by changes in G-C percentage and length.

25 While the invention has been described with reference to selection of sequences which are suitable targets for the design of antisense oligonucleotides, it will be appreciated that the methods described herein can be used to identify regions of a target nucleic acid sequence (including, but not limited to, a coding or non-coding DNA or  
30 RNA) which are suitable for interaction with other ligands which can bind to the nucleic acid, including one or more of: a compound which binds to a nucleic acid in a sequence-specific way (e.g., a sequence specific cleavage enzyme, such as a restriction endonuclease, including EcoRI, HaeIII, BamHI and BglI, or an enzyme or other molecule which binds to a specific sequence, e.g., molecules which modulate the  
35 expression of a product encoded by a nucleic acid) or in a sequence-non-specific way (e.g., DNaseI or micrococcal nuclease); a protein; an enzyme; an enzyme or other

molecule (and agonists or antagonists thereof) which alters the structure of a nucleic acid to which it binds, e.g., by breaking or forming a covalent or non-covalent bond, e.g., a hydrogen bond, between an atom of the nucleic acid and another atom, e.g., an atom of the same strand, an atom of the complementary sequence, or an atom of another molecule; an enzyme which cleaves one or both strands of the nucleic acid, and agonists or antagonists thereof; an enzyme which methylates or alkylates the nucleic acid, and agonists or antagonists thereof; an enzyme which promotes or catalyzes the synthesis of a nucleic acid, e.g., a polymerase which requires a double stranded primer, and agonists or antagonists thereof; a DNA polymerase, e.g., DNA polymerase I or Taq polymerase, and agonists or antagonists thereof; an enzyme which alters the primary or secondary structure of a nucleic acid, e.g., a topoisomerase, or an enzyme related to recombination or replication, and agonists or antagonists thereof; a DNA binding ligand, and agonists or antagonists thereof; a mutagen; a compound which enhances gene expression, and agonists or antagonists thereof; a compound which intercalates into a double stranded nucleic acid, and agonists or antagonists thereof; a compound which, when contacted with a reaction mixture comprising a first single stranded nucleic acid and a second single stranded nucleic acid will accelerate the rate of duplex formation at least  $n$ -fold, wherein  $n$  is an integer between 2 and 1,000, inclusive; a compound which will decrease the free energy of duplex formation by  $n$ -fold, wherein  $n$  is an integer between 1 and 1,000 inclusive; a small molecule, e.g., any metalloorganic compound, any heterocyclic compound, or any protein which binds a nucleic acid; proteins or other molecules which are associated with the structural organization of DNA in the cell nucleus, or the packaging of DNA, including histones and nucleosomes; nucleic acid binding mutagens or carcinogens, or agonists or antagonists thereof; viral proteins and agonists or antagonists thereof. Thus, the methods of the invention have broad applicability.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

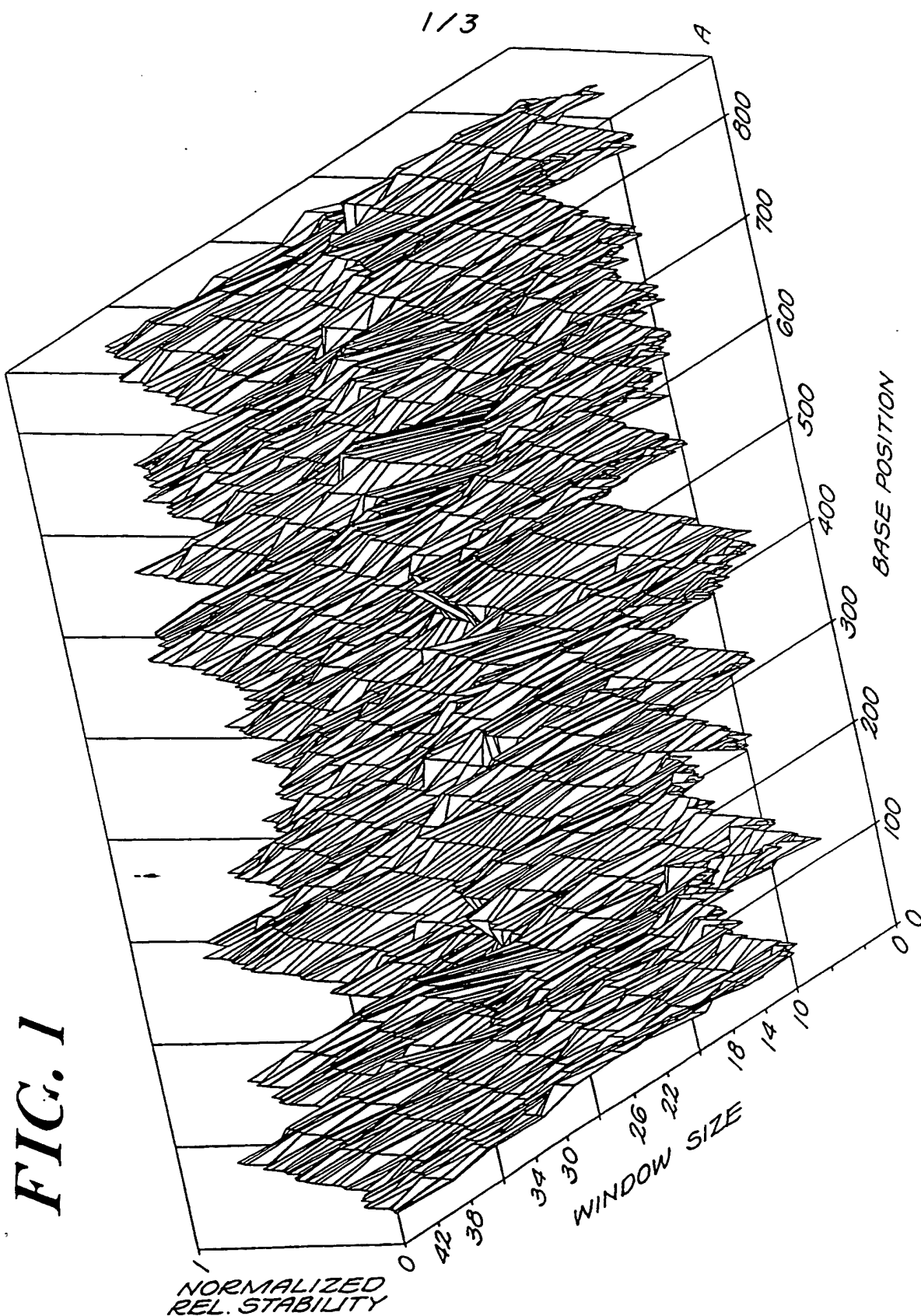
The contents of all references and patent applications cited herein are hereby incorporated by reference.

Other embodiments are within the following claims.

- 10 -

What is claimed is:

1. A method of identifying a site on a nucleic acid sequence having a desired free energy variability comprising:
  - 5 providing a nucleotide sequence of length Z;
  - calculating the free energy of a plurality of windows centered on a base pair for a plurality of base pairs from the nucleotide sequence, wherein the number of window sizes is between 2 and Y, wherein Y is an integer between 3 and 100;
  - for each window size, constructing a free energy distribution along the sequence,
  - 10 normalizing the distribution to a standard scale;
  - determining the mean normalized free energy values for all windows for each base pair position;
  - subtracting the mean value for a position and providing the deviation from the mean of each base position to determine a site having the desired free energy variability.
- 15 2. The method of claim 1, wherein the plurality of base pairs comprises at least 50% of the base pairs of the nucleotide sequence.
3. A method of identifying an optimized ligand binding site on a nucleic acid
  - 20 sequence, the method comprising:
  - providing a nucleic acid sequence;
  - calculating a free energy value for at least two window sizes at each of a plurality of base pairs of the nucleic acid sequence;
  - normalizing the free energy values for each window size at each base pair to a
  - 25 standard scale; and
  - calculating a deviation of each normalized free energy value at a base pair from a mean normalized free energy value at the base pair; and
  - selecting a base pair at which a large deviation from the normalized free energy value is calculated, relative to at least one other base pair;
  - 30 such that an optimized ligand binding site on the nucleic acid sequence is identified.



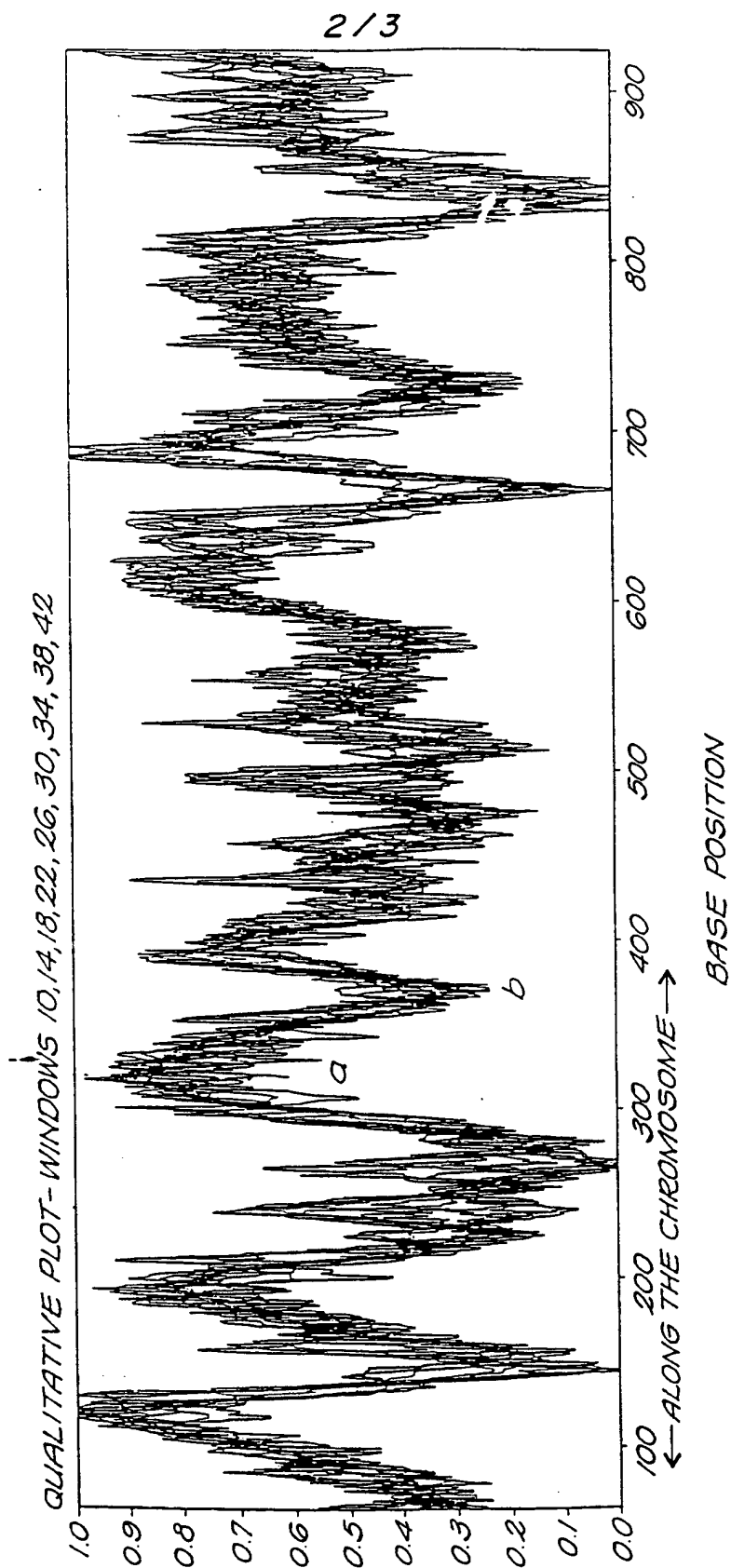


FIG. 2

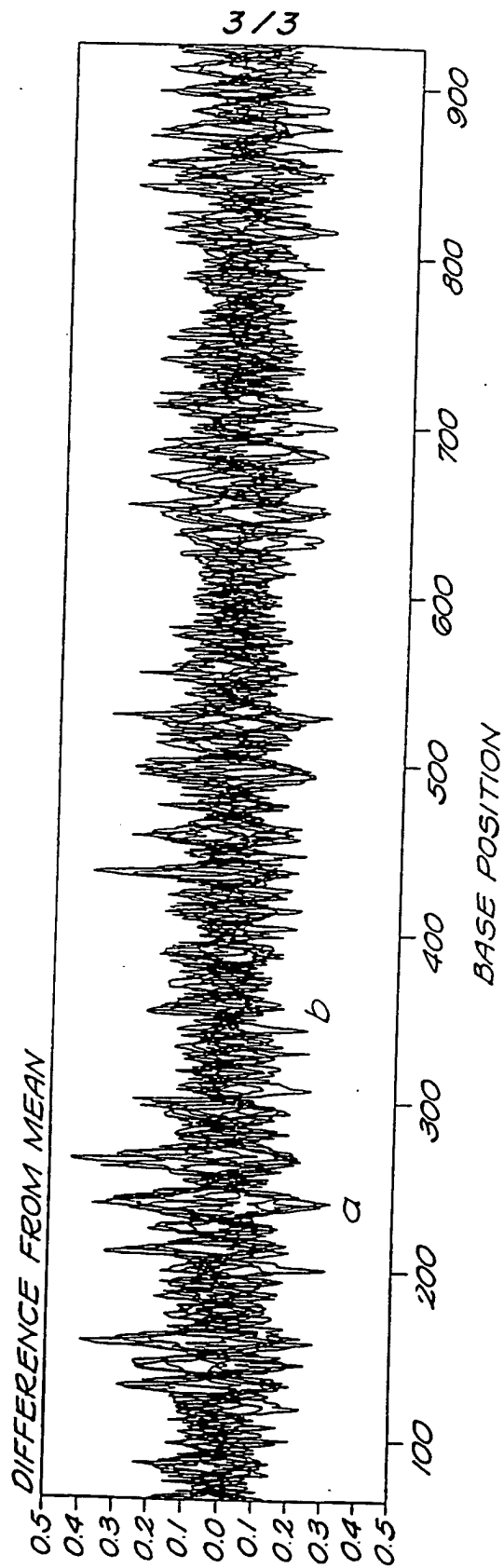


FIG. 3

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/03726

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68

US CL : 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog: Medline, BIOSIS, CAS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,496,698 A (DRAPER ET AL.) 05 March 1996, see entire document.	1-3
A	US 5,525,468 A (MCSWIGGEN) 11 June 1996, see entire document.	1-3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

02 JUNE 1998

Date of mailing of the international search report

23 JUN 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

John LeGuyader

Telephone No. (703) 308-0196